

In-vitro cytotoxicity study of methanolic fraction from *Ajuga Bracteosa* wall ex. benth on MCF-7 breast adenocarcinoma and hep-2 larynx carcinoma cell lines

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ABSTRACT

Objective: *Ajuga bracteosa* Wall ex Benth (Labiateae) is popularly known in India as "Neelkanthi." A decoction of the leaves, flowers, and barks is used in India for the treatment of cancer including diabetes, malaria, and inflammation etc. The main objective of this study is to investigate the cytotoxic potential of *Ajuga bracteosa*. **Materials and Methods:** Successive solvent extraction of *Ajuga bracteosa* in petroleum ether, methanol, and water extracts was done. These extracts were tested against human breast adenocarcinoma (MCF-7) and larynx carcinoma (Hep-2) tumor cell lines, using the thiazolyl blue test (MTT) assay. **Results:** The methanolic fraction of *Ajuga bracteosa* had shown the significant results against MCF-7 and Hep-2 tumor cell lines. The methanolic, petroleum ether and aqueous extract from *Ajuga bracteosa*, presented an IC₅₀ value at 24 h of 10, 65, 70 µg/ml and 5, 30, 15 µg/ml on MCF-7 and Hep-2 cells, respectively. Steroids compounds namely β-sitosterol and unknown constituents were identified in the most active methanol extract of *Ajuga bracteosa* wall ex Benth. These known and unknown compounds exhibited cytotoxic potential against MCF-7 and Hep-2 cancer cells. **Conclusion:** Among all the tested extracts, methanolic extract can be considered as potential sources of anti-cancer compounds. Further studies are necessary for more extensive biological evaluations.

Key words: *Ajuga bracteosa*, cytotoxicity studies, Hep-2, MCF-7, MTT assay

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INTRODUCTION

Cancer is an ailment that affects more or less 200 types of cells. The major characteristic is the lack of control of the cell proliferation, differentiation, and death including invading organs and tissues. There are many difficulties in the treatment, but the more frequently are the drug resistance, toxicity, and low specificity.^[1]

The use of natural products as anti-cancer agents has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine. Several drugs currently used in chemotherapy were isolated from plant species or derived from a natural prototype. They include the vinca alkaloids as vinblastine

and vincristine, which is isolated from *Catharanthus roseus*, etoposide and teniposide are the semi-synthetic derivatives of epipodophyllotoxin, which is isolated from species of the genus Podophyllum, the naturally derived taxanes isolated from species of the genus Taxus, the semi-synthetic derivatives of camptothecin, irinotecan and topotecan, isolated from *Camptotheca acuminata*, and several others.^[2] According to Cragg and Newman,^[3] over 50% of the drugs in clinical trials for anti-cancer activity were isolated from natural sources or are related to them.

The aerial part of *Ajuga bracteosa* Wall ex Benth known also as Neelkanthi, which belongs to the Labiateae family, has been used as either a food material or a traditional oriental medicine. The extracts from *Ajuga bracteosa* have been reported to have a wide range of health benefits. Particularly, in India, the leaves grown for 4 years have been used to treat diuretic, gout, malarial fever,^[4] blood-purifier, and inflammatory diseases.^[5] Some active compounds, such as β-sitosterol^[6] and phenolic components,^[7] have been identified. Some studies reported that its alkaloidal

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fraction showed stimulant action and the powder is used for burns.^[8]

The search for new drugs exhibiting activity against several types of cancer is one of the most interesting subjects in the field of natural products research. The aim of the present work is to investigate *In vitro* cytotoxic potential of *Ajuga bracteosa* (aerial part) plant used in traditional medicine against MCF-7 and Hep-2 cancer cells.

MATERIALS AND METHODS

Plant material

The plant of *Ajuga bracteosa* was identified and collected in January from Hamirpur district of Himachal Pradesh. The aerial parts of *Ajuga bracteosa* was authenticated by Botanist Dr. Zia Ul Hassan at Department of Botany, Safia Science College, Bhopal, Madhya Pradesh. The Voucher specimen No. is 131/Bot/Safia/2010.

Extraction procedure

The dried aerial parts of *Ajuga bracteosa* (100 g) were cut into small pieces, powdered, and then extracted with methanol^[9] for 36 hrs. The viscous semi-solid extract was collected in a tared conical flask the solvent was removed by distillation and last traces of solvent being removed under vacuum. The yield was found 15.45 g for methanolic fraction.

Phytochemical screening

Methanolic extract of *Ajuga bracteosa* was subjected to qualitative tests as Liebermann-burchard test and solkowski reaction for the identification of sterols (β -sitosterol), Turbidity test, and ferric chloride test for the confirmation of resins present in this plant.^[10]

Chromatographic studies

Thin-layer chromatography

Out of the various solvent systems tried, benzene: ethyl acetate (9.9:0.1) showed the best resolution and spots for methanolic extract of *Ajuga bracteosa*. The detecting reagent was 50% methanolic sulfuric acid followed by heating at 110°C for 5 min.^[11]

High performance thin layer chromatography (HPTLC) study

Selection of HPTLC plates pre-coated and pre-activated TLC plates (E. Merck No. 5548) of silica gel 60 G F_{254 + 366}, with the support of aluminum sheets having thickness of 0.1 mm and size 20 × 20 cm, were cut smaller according to required dimensions.^[12]

Preparation of standard stock solution

β -sitosterol (1 mg) dissolved in 1 ml of solvent was used for sample preparation, and 10.0 μ l of sample

were applied as band length 15.0 mm from applied position, of the plate 100 μ l syringe on automatic sample applicator (LINOMAT-V, CAMAG COMPANY, SWITZERLAND).

Preparation of sample extracts stock solutions

20 mg of sample methanolic fraction was dissolved in 1 ml of methanol. 10 μ L each of the different concentration of sample extracts were spotted (band with: 30 mm) in triplicate on HPTLC plates pre-coated with 0.2 mm layer of Si-gel Si60GF254 (E. Merck) using an automatic applicator Linomat-5. Chromatograms were developed for 9.0 cm using mobile phase n-hexane: ethyl acetate (4:1v/v).

Chromatography was performed in a twin trough chamber (Camag, Switzerland). After development, the plates were sprayed with 50% methanolic sulfuric acid reagent and the spots were detected by heating the plate at 120°C for 20 min. The developed plates were then scanned at 254 nm so as to record the peak areas. The calibration curve of methanolic fraction stock solution was obtained by plotting peak area vs. concentration.

Application of sample

The extract samples were applied in the form of a band using CAMAG LINOMAT V, an automatic sample application device, maintaining a band width 9 mm, space 9 mm, 15 μ L/sec. The quantity of sample applied was 5-10 μ L.^[13]

HPTLC development

The developed mobile phase was selected experimentally as n-hexane: ethyl acetate (4:1). The plates were developed by placing in a pre-saturated tank (12 cm height) with the mobile phase for 2 hours. The plates were dried by evaporating the solvent either at room temperature or by spraying hot air by air dryer. The HPTLC densitograms are shown in Figure 1 and 2.

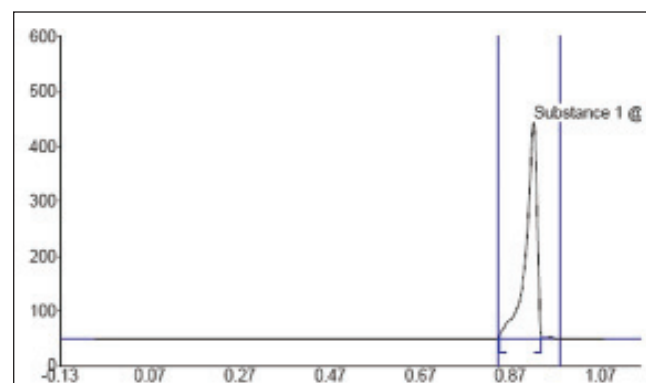


Figure 1: HPTLC chromatogram of β -sitosterol (standard sample) at 254 nm

Test sample preparation

Stock solutions of the methanolic extract of *Ajuga bracteosa* test samples were prepared at concentration of 100 mg/ml in dimethyl sulfoxide (DMSO). A serial two-fold dilution was then made from the stock solution in DMSO to produce working solutions of 0.01, 0.1, 1, 10, and 100 µg/ml as well as solutions of 0.1% DMSO in complete culture were used as the vehicle control.

Cell culture conditions

MCF-7 (breast adenocarcinoma) cells were grown in minimal essential medium supplemented with glutamine (0.6 g/l) [Jinan Jiaquan Chemical Co. Ltd., Bombay Harbor], gentamicin (25 mg/ml) [Anhui Minmentals Dev. Imp. and Exp. Co. Ltd., Japan] and fetal calf serum [Zen Biotech Pvt. Ltd., Hyderabad] and HEp-2 (larynx carcinoma cells) were grown in minimal essential medium supplemented with L-glutamine (0.1 g/l), sodium bi carbonate (2.2 g/l) [Shandong Lukang Record Pharmaceuticals Ltd.], non-essential amino acid (10 ml/l) [Archon Vitamin Corporation, Ievington, New Jersey], and

fetal calf serum. Cells were cultured at 37°C in a humified 5% CO₂ atmosphere, and conserved in a log growth phase.

In-vitro cytotoxic activity assay

This assay was performed according to a slight modification of the procedure reported by Mosman.^[14] In experiment, cells were plated in 96-well plates (10⁵ cells/well for adherent cells or 0.3 × 10⁶ cells/well for suspended cells in 100 µl of medium). This assay was performed at Deshpande Laboratories, Bhopal using the standard operating procedures. After 24 hrs, methanolic fractions of *Ajuga bracteosa* were dissolved in DMSO and serially diluted with complete medium to get the concentrations a range of test concentration (0.01, 0.1, 1, 10, and 100 µg/ml). DMSO concentration was kept less than 0.1% in all the samples. Prepared dilutions were added to different wells, and cells were incubated for 96 hrs. Control groups received the same amount of DMSO. Growth of tumoral cells was quantified by ability of living cells to reduce the yellow dye 3- (4, 5-dimethyl-2- thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product. At the end of 96 hrs incubation, the medium in each well was replaced by fresh medium containing 0.5 mg/ml of MTT. After 4 hrs, the formazan product of MTT reduction was dissolved in DMSO and absorbance was measured at 550 nm. Drug effect was quantified as the % of control absorbance of reduced dye at 550 nm. The experiments were performed in triplicate.

%Cytotoxicity =

$$\frac{\text{Absorbance of cell without treatment} - \text{Absorbance of cell with treatment}}{\text{Absorbance of cell without treatment}} \times 100$$

Statistical analysis

The IC₅₀ values are calculated for different concentrations of MCF7- breast adenocarcinoma, Hep-2 larynx carcinoma cell lines and compared statistically with the control.

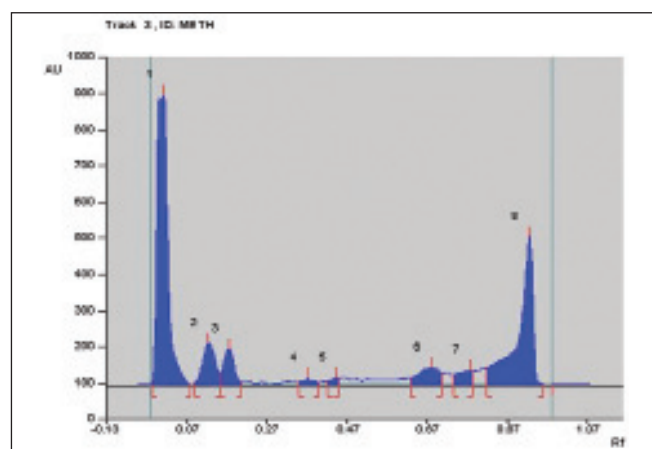


Figure 2: HPTLC chromatogram of methanolic fraction of *Ajuga bracteosa* at 254 nm

Table 1: HPTLC profile of β-sitosterol (standard sample) at 254 nm

Peak	Start R _f	Start height	Max R _f	Max height	Max %	End R _f	End height	Area	Area %	Concentration (µg/ml)
1	0.85	2.4	0.92	394.0	100.0	0.94	1.9	7054.8	100.0	10

Table 2: HPTLC profile of methanolic fraction of *Ajuga bracteosa* at 254 nm

Peak	Start R _f	Start Height	Max R _f	Max height	Max %	End R _f	End height	Area	Area %	Conc. In (µg/ml)
1	-0.02	4.7	0.01	799.2	29.82	0.08	0.2	15510.9	46.42	7.17
2	0.09	3.7	0.13	114.8	4.13	0.15	32.2	2568.8	7.69	1.18
3	0.15	32.2	0.18	99.6	4.84	0.21	6.2	1827.3	5.47	0.84
4	0.35	6.4	0.37	17.2	2.58	0.40	5.8	313.7	0.94	0.145
5	0.42	8.4	0.45	18.7	3.00	0.45	16.1	264.8	0.79	0.122
6	0.63	16.8	0.68	47.1	4.83	0.71	31.3	1776.0	5.32	0.821
7	0.74	27.2	0.78	38.6	3.78	0.78	36.2	1005.4	3.01	0.465
8	0.82	44.4	0.93	407.9	4.46	0.96	0.2	10145.3	30.36	4.69

RESULTS

Preliminary phytochemical study of methanolic extract of *Ajuga bracteosa* showed the presence of sterols, resins, and other unknown compounds. After preliminary TLC detection and development of suitable mobile phase n-hexane: Ethyl-acetate (4:1), HPTLC analysis was carried out for tracing β -sitosterol in the methanolic fractionate of *Ajuga bracteosa*. The HPTLC profile of β -sitosterol (Standard sample) showed the single spot with R_F 0.92 and concentration 10 $\mu\text{g/ml}$ or the maximum percentage of area is 100% [Table 1 and Figure 1]. The HPTLC profile of methanolic extract of *Ajuga bracteosa* revealed eight spots with R_F of 0.01, 0.13, 0.18, 0.37, 0.45, 0.68, 0.78, and 0.93 (β -sitosterol) and concentration 7.17, 1.18, 0.845, 0.145, 0.122, 0.821, 0.465, and 4.690 $\mu\text{g/ml}$ or the maximum percentage of area is 30.36% [Table 2 and Figure 2].

The methanolic fraction of *Ajuga bracteosa* showed IC_{50} lower than 5 $\mu\text{g/ml}$ against the Hep-2 and 10 $\mu\text{g/ml}$ against the MCF-7 cell lines. However, according to the criteria of the American National Cancer Institute, the IC_{50} limit to consider a crude extract promising for further purification is lower than 30 $\mu\text{g/ml}$.

The significant cytotoxic activity was detected for the methanolic fraction of *Ajuga bracteosa* (aerial part) presenting IC_{50} values lower than 5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ against two cell lines (MCF-7 and Hep-2) [Table 3 and Figure 3 and 4].

The result of the present study indicates the presence of β -sitosterol and other compounds in the methanolic fraction may be responsible for anti-cancer activity.

DISCUSSION

The search for anti-cancer agents from natural sources has been successful worldwide; active constituents have

been isolated and are nowadays used to treat human tumors. The ethnopharmacological knowledge is helpful to lead the search for plants with potential cytotoxic activity.^[15]

The present study was undertaken to evaluate the cytotoxic activity of *Ajuga bracteosa*, the traditional medicines, that are used in the treatment of cancer and cancer-related illnesses in the country. Ethnopharmacological data (information based on the medicinal traditional use of plants) has been one of the common useful ways for the discovery of biologically active compounds from plants.^[16] The advantage of the ethnopharmacological information is that the extensive literature may already allow for some rationalization with respect to the biological potential of a reputed use.^[17]

On the basis of reported traditional uses, this plant *Ajuga bracteosa* is selected for the study of the anti-cancer activity. In this study, methanolic extract of *Ajuga bracteosa* was used for the treatment of cancer (supposed to contain mainly semi-polar compounds). Traditional healers who were interviewed on how they prepare the extracts before administering to the patients indicated that it was the water decoction that was administered, meaning that it is the polar compounds that were responsible for the reported anti-cancer activity.

Table 3: In-vitro cytotoxicity of methanolic fraction from *Ajuga bracteosa* wall ex Benth. on MCF-7 and Hep-2 cell lines measured by the MTT assay

Treatment	Cell line IC_{50} ($\mu\text{g/ml}$)	
	MCF-7	HEP-2
Methanolic extract	10	5

The IC_{50} and its 95 % confidence interval (CI 95%) were obtained by non-linear regression. ND: Not determined. Extracts with an IC_{50} value lower than 10 $\mu\text{g/ml}$ were considered active

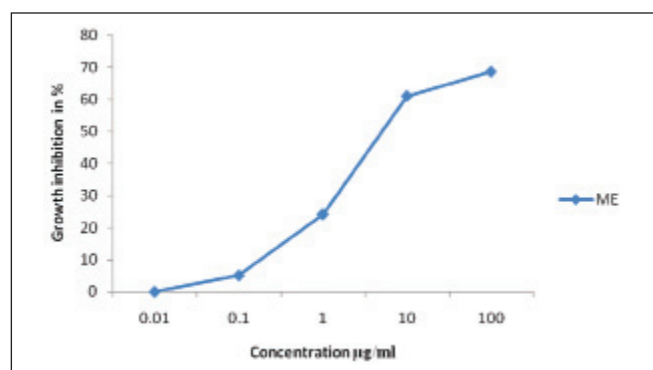


Figure 3: Percentage — Inhibition curve of methanolic extract of *Ajuga bracteosa* for cytotoxic activity. Percentage inhibition plotted against the concentration of methanolic fraction against MCF-7 breast adenocarcinoma cell-line

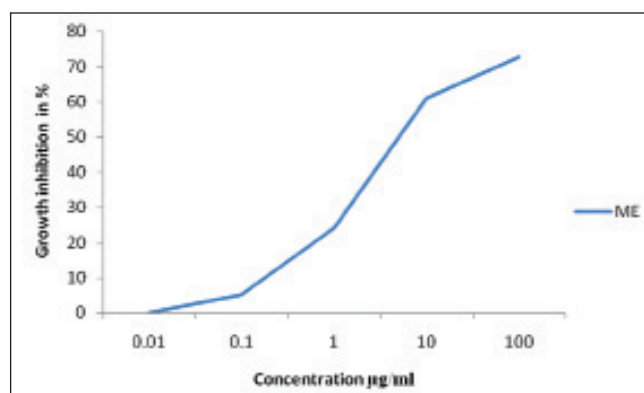


Figure 4: Percentage — Inhibition curve of methanolic extract of *Ajuga bracteosa* for cytotoxic activity. Percentage inhibition plotted against the concentration of methanolic fraction for Hep-2 larynx carcinoma cell line

An anti-cancer activity of methanolic extract of *Ajuga bracteosa* was investigated using a MTT assay on two human cancer cell lines, MCF-7 and Hep-2. A mitochondrial enzyme in living cells, succinate-dehydrogenase cleaves the tetrazolium ring and converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells, according to Mosmann,^[14] shows that methanol extract significantly inhibited cancer cell growth at a concentration of 100 µg/ml due to the presence of compounds in the extract.

CONCLUSION

In conclusion, *In vitro* cytotoxic potential of *Ajuga bracteosa* (aerial part) plant used traditionally as folklore was confirmed to exhibit antioxidant and anti-cancer activity. Probably, β -sitosterol and unknown compounds as resins in this study traced out by preliminary phytochemical screening, TLC and HPTLC technique are responsible (individually or collectively) to work against MCF-7 and Hep-2 cancer cells.

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